Sumoylation of Pdx1 is associated with its nuclear localization and insulin gene activation

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Kishi, Akio, Takaaki Nakamura, Yoshihiko Nishio, Hiroshi Maegawa, and Atsunori Kashiwagi. Sumoylation of Pdx1 is associated with its nuclear localization and insulin gene activation. Am J Physiol Endocrinol Metab 284: E830–E840, 2003. First published December 17, 2002; 10.1152/ajpendo.00390.2002.—Pancreatic duodenal homeobox-1 (Pdx1) is a transcription factor, and its phosphorylation is thought to be essential for activation of insulin gene expression. This phosphorylation is related to a concomitant shift in molecular mass from 31 to 46 kDa. However, we found that Pdx1 was modified by SUMO-1 (small ubiquitin-related modifier 1) in β-TC-6 and COS-7 cells, which were transfected with Pdx1 cDNA. This modification contributed to the increase in molecular mass of Pdx1 from 31 to 46 kDa. Additionally, sumoylated Pdx1 localized in the nucleus. The reduction of SUMO-1 protein by use of RNA interference (SUMO-iRNAs) resulted in a significant decrease in Pdx1 protein in the nucleus. A 34-kDa form of Pdx1 was detected by the cells exposed to SUMO-iRNAs in the presence of lactacystin, a proteasome inhibitor. Furthermore, the reduced nuclear sumoylated Pdx1 content was associated with significant lower transcriptional activity of the insulin gene. These findings indicate that SUMO-1 modification is associated with both the localization and stability of Pdx1 as well as its effect on insulin gene activation.

The marked change in molecular mass of Pdx1 led us to suspect that the addition of ubiquitin could account for the 15-kDa difference. Thus we speculate that other posttranslational modification may be attributed to the increase in molecular mass of Pdx1 and regulate its function.

Pancreatic duodenal homeobox-1 (Pdx1) is a transcription factor encoded by a homeobox gene (27, 33, 34). In humans and other animal species, the embryonic development of the pancreas requires Pdx1. This role for Pdx1 is supported by the finding of a mutant Pdx1 in humans and other animal species, the embryonic development of the pancreas requires Pdx1. This role for Pdx1 is supported by the finding of a mutant Pdx1 in Pdx1 is associated with its nuclear localization and insulin gene activation. For Pdx1 to exert its transcriptional activity, it must translocate from the cytoplasm to the nucleus. This process involves intracellular signaling through either phosphatidylinositol 3-kinase (PI 3-kinase) or stress-activated protein kinase (SAPK2/p38) pathways (8, 20, 37, 51). The participation of either pathway leads to phosphorylation of Pdx1, and then Pdx1 enters the nucleus. Stimulation of nuclear translocation of Pdx1 by either glucose or insulin is inhibited by either wortmannin, a PI 3-kinase inhibitor, or SB-203580, a SAPK2 inhibitor. These findings suggest the participation of both PI 3-kinase and SAPK2/p38. Furthermore, exposure of recombinant Pdx1 protein to extract from pancreatic β-cells enhances its DNA-binding activity. This change is linked to a shift in molecular mass from 31 to 46 kDa (20). Many studies also indicate that the molecular mass of Pdx1 extracted from cultured pancreatic β-cells lines at various conditions is estimated to be ~46 kDa (37, 42, 47, 48). In addition, it is also reported that there is a tissue-specific phosphorylation pattern of Pdx1 between pancreatic endocrine and duct cells (11). However, it is unlikely that phosphorylation of Pdx1 is responsible for the 15-kDa difference. Thus we speculate that other posttranslational modification may be attributed to the increase in molecular mass of Pdx1 and regulate its function.

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possibility that the addition of SUMO-1 to Pdx1 causes a shift of the molecular mass from 31 to 46 kDa to facilitate the localization and function of Pdx1.

MATERIAL AND METHODS

Plasmids, SUMO-1 RNA interference, recombinant adenoviruses, and recombinant Pdx1 protein preparation. The complete coding sequence of mouse Pdx1 was inserted separately in the plasmid vector pcDNA3.1 (Invitrogen, Carlsbad, CA) to yield pcDNA-Pdx1 according to a procedure previously described (49). For the construction of the histidine (His)-tagged SUMO-1 expression vector, the full sequence of human SUMO-1 cDNA was amplified by PCR with specific primers (sense primer 5′-ATGCATCACCACCACATCCTGACCAGGGGGA, antisense primer 5′-TTTCAAGAGATGGGCTTGC) and subcloned into EcoRI (TAKARA, Shiga, Japan) sites of the pcDNA3.1 vector. The 374-bp (−362 to 12) fragment of the rat insulin 1-promoter gene was cloned with the primers 5′-ACCGTGTTACGGTGCTCCCAA-CAACGC (sense primer) and 5′-CTCGAGTTAGGTTGAGGGTAGTTACTGG (antisense primer) using genomic DNA as a template and subcloned into MluI and XhoI (TAKARA) sites of pGL3-basic luciferase vector (Promega, Madison, WI) to yield pINS-Luc.

To construct the double strand of SUMO-RNA interference (iRNA), 21 nucleotides for SUMO-1 (sense 5′-UAUAUAUUAAACUCATT, antisense 5′/H11032/ H11032/UAUUCACCTT) were chemically synthesized using RNA phosphorimidites and thymidine phosphoramidite (Japan Bio-Service, Saitama, Japan). iRNAs (50 μM) of both sense and antisense strands were dissolved and annealed in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, and 2 mM magnesium acetate) for 1 min at 90°C, followed by 1 h at 37°C.

cDNA encoding the complete coding sequence of mouse Pdx1 was subcloned in pACCMV-pLpA vector and cotransformed with pJM17 plasmid into 293 cells (6). Successful homologous recombination between these two plasmids resulted in recombinant viruses encoding the specific genes (Ad-Pdx1 and Ad-β-gal). After the plaque purification, viruses were amplified in 293 cells and plaque-forming units (PFU) were assayed using the Adeno-X rapid titer kit (Clontech, Palo Alto, CA). Adenoviral titrations were 3.4 × 109 PFU/ml for Ad-Pdx1 and 109 PFU/ml for Ad-β-gal, respectively.

To obtain recombinant Pdx1 protein in Escherichia coli, the full sequence of mouse Pdx1 was inserted in BamHI and KpnI (TAKARA) sites of pQE-100 vector (QIAGEN, Valencia, CA) to yield His-tagged Pdx1 protein.

Cell culture and transfection of expression vector or SUMO-iRNAs. COS-7 cells (kidney fibroblast cell line from African green monkey, passages 20–25), HEK-293 cells (kidney epithelial cell line from Homo sapiens), and β-TC-6 cells (insulinoma cell line from mouse) were purchased from American Type Culture Collection (Rockville, MD) and cultured continuously in DMEM supplemented with 10% heat-inactivated FBS, 25 mM n-glucose, 44.0 mM NaHCO3, 1 g/l streptomycin, and 10 μg/ml penicillin G at 37°C under a humidified atmosphere of 95% air–5% CO2. Treatment was performed using the previous method (31). In brief, COS-7 cells were transfected with 5 μg pcDNA-Pdx1, His-tagged SUMO-1 pcDNA expression vector, and pINS-Luc vector using the Lipofectamine transfection reagent (Invitrogen). After a 48-h incubation, the cells were washed twice with PBS and analyzed for molecular biological analysis. Transfection of 0.6 nmol single-interfering RNA duplexes (iRNAs) for targeting endogenous SUMO-1 genes in both β-TC-6 and COS-7 cells was also carried out using the Lipofectamine transfection reagent.

Treatment of proteasome inhibitor lactacycin. First, COS-7 cells were transfected with SUMO-iRNAs for 48 h, and then the cells were transduced with Ad-Pdx1 and incubated for 48 h with 10 μM lactacycin (Calbiochem-Novabiochem, San Diego, CA) diluted in DMSO (Sigma-Aldrich, St. Louis, MO), which was a proteasome inhibitor. The control cells were incubated with only DMSO for 48 h. Medium containing DMSO or lactacycin was changed every 12 h.

RNA extraction and Northern blot analysis. Total RNA was extracted from the cells using a procedure described previously (52). Aliquots (10 μg) of each sample of total RNA were used for Northern blot analysis. The transferred blots were hybridized with a 32P-labeled fragment of Pdx1 cDNAs. The signal arising from 18S ribosomal mRNA served as the internal control to evaluate differences among various samples.

Preparation of nuclear extracts and Western blot analysis. Preparation of nuclei and cytosolic proteins was performed as described previously (13). Briefly, cytosolic extracts were collected in hypotonic HEPES buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, and 1 mM dithiothreitol) containing protease inhibitors and Nonidet P-40 (NP-40, 0.5%) for 20 min at 4°C. Nuclear extracts were collected with hypertonic HEPES buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, and 1 mM dithiothreitol) and agitated for 10 min on ice, followed by 1 h at 37°C.

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isolated subcellular fractions from the molecular mass of endogenous Pdx1 protein, we
found that a shift in the apparent molecular mass of Pdx1 was previously attributed to phosphorylation (20). However, we speculated that phosphorylation is an unlikely explanation of such a large difference.

Posttranslational modification of Pdx1. To determine the molecular mass of endogenous Pdx1 protein, we isolated subcellular fractions from β-TC-6, a pancreatic β-cell line. The nuclear and cytoplasmic extracts were investigated by Western blot analysis using an anti-Pdx1 antibody (Fig. 1A). Endogenous Pdx1 protein in β-TC-6 cells was found predominantly in the nuclear fraction and had a molecular mass of 46 kDa. The abundance of Pdx1 was not affected by the concentration of glucose in medium. Others have suggested (20) that a shift in the apparent molecular mass of Pdx1 from 31 to 46 kDa was involved in nuclear translocation of the protein in MIN6 cells, but we could not detect the lower molecular mass form of Pdx1 in β-TC-6 cells.

It is possible that the 31-kDa form of Pdx1 may be the unmodified form of the protein. We tested this possibility by inserting the full-length mouse Pdx1 (284 amino acids) in pQE vector and expressing it in bacteria. Consistent with this idea, this unmodified form of Pdx1 had the predicted molecular mass of 31 kDa (Fig. 1B, lane 4). To further demonstrate that the modified form of Pdx1 had a molecular mass of 46 kDa, we overexpressed mouse Pdx1 in a mammalian cell line (COS-7). The Pdx1 in COS-7 cell lysates transfected with the cDNA-inserted Pdx1 had a molecular mass of 46 kDa (Fig. 1B, lane 2).

The difference of 15 kDa between the two forms of Pdx1 was previously attributed to phosphorylation (20). However, we speculated that phosphorylation is an unlikely explanation of such a large difference.

**RESULTS**

**A**

- **IP : SUMO-1**
  - **IB : Pdx1**
  - **46 kDa**

**B**

- **IP : SUMO-1**
  - **IB : Pdx1**
  - **46 kDa**

**C**

- **IP : His**
  - **IB : Pdx1**
  - **46 kDa**

**Fig. 2.** A: immunoprecipitation analysis with small ubiquitin-related modifier (SUMO)-1 antibody using cytoplasmic (C) and nuclear (N) extracts from β-TC-6 cells incubated with DMEM containing 2.5 or 25.0 mM glucose. B: immunoprecipitation analysis with SUMO-1 antibody using cytoplasmic and nuclear extracts from COS-7 cells transfected with pcDNA-Pdx1. C: cells were transfected with His-tagged SUMO-1 expression vector (His-SUMO-1) and/or pcDNA-Pdx1. The extracts were immunoprecipitated with His antibody and analyzed by immunoblotting with anti-Pdx1 antibody. The positions of molecular mass are on right. IP, immunoprecipitation; IB, immunoblotting.
Nevertheless, we had to rule out the possibility that phosphorylation accounted for the changes in the molecular mass of Pdx1. Therefore, we examined Pdx1 protein expressed in COS-7 cells and measured its molecular mass in the presence of phosphatase (Fig. 1B) according to the method described previously (20). A 31-kDa form of Pdx1 was not detected in the presence of phosphatase, and exposure of Pdx1 to phosphatase.

![Fig. 3. A: Western blot analysis of Pdx1 and SUMO-1 in the extracts from COS-7 cells transduced with Ad-Pdx1 in the absence or presence of SUMO-RNA interference (iRNAs). Whole cell extracts were prepared from cells transduced with Ad-β-gal (lanes 1 and 2) and Ad-Pdx1 (lanes 3 and 4) in the absence of SUMO-iRNAs. Lanes 5 and 6 show the results of Western blot analysis using COS-7 cell extracts with Ad-Pdx1 in the presence of SUMO-iRNAs. The abundance of β-actin reflects an equal loading of proteins in each lane. The blot shown is 1 representative of 3 independent experiments with a different sample in each lane. The positions of molecular mass are on right. B: data from densitometric analysis of the expression of both Pdx1 and SUMO-1. Values are shown as means ± SE (n = 6). #P < 0.01 and *P < 0.05 vs. the values for COS-7 cell extracts transfected with SUMO-iRNAs.]

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tase caused a slight lowering of the molecular mass (Fig. 1B, lane 3). These results revealed the following significant points: 1) unmodified Pdx1 had a molecular mass of 31 kDa, 2) modified Pdx1 had a molecular mass of 46 kDa, 3) Pdx1 was phosphorylated, and 4) phosphorylation of Pdx1 was not the sole posttranslational modification of the protein.

Pdx1 protein modification by covalent attachment of SUMO-1. We wondered whether the 46-kDa form of Pdx1 might be modified by SUMO-1. To test this possibility, we immunoprecipitated endogenous Pdx1 from β-TC-6 cells and exogenous Pdx1 from COS-7 cells using anti-SUMO-1 antibody. Both nuclear and cytoplasmic extracts from these cells were analyzed using immunoprecipitation with the Pdx1 antibody and the anti-mouse SUMO-1 or anti-His-tagged antibodies (Fig. 2, A–C). Negative controls were prepared from COS-7 cells transfected with empty vector (Fig. 2, B and C). As shown in Fig. 2, A and B, a single Pdx1-reactive band was found in the nuclear fraction with molecular mass of ~46 kDa. In addition, the identical band cross-reacted with anti-His-tagged antibody (Fig. 2C) in COS-7 cells transfected with pcDNA-Pdx1- plus His-tagged-SUMO-1 expression vector. None of these bands was detected in cells transfected with empty vector alone (Fig. 2B) or with His-tagged-SUMO-1 expression vector (Fig. 2C). Furthermore, because the SUMO family consists of SUMO-1, SUMO-2, and SUMO-3 (19, 53), we also tested whether SUMO-2 and SUMO-3 potentially interacted with Pdx1 by immunoprecipitation methods using anti-SUMO-3 antibody, which cross-reacts with both SUMO-3 and SUMO-2. However, we did not observe SUMO-3- and/or SUMO-2-reactive bands by the immunoprecipitation procedure (data not shown). These findings suggest that the Pdx1 protein found in the nucleus was modified by covalent attachment to SUMO-1 in both pancreatic and nonpancreatic cells.

Effects of SUMO-iRNAs on expression of Pdx1. Uptake of double-strand RNA by insect cell lines causes knockdown expression of specific genes, resulting from sequence-specific mRNA degradation (1, 7, 10), so-called iRNA. On the basis of the findings above, we knocked down SUMO-1 expression and analyzed its effect on both function and stability of Pdx1. We used a 21-nucleotide RNA duplex (SUMO-iRNAs) to knock down endogenous expression of the SUMO-1 gene. The COS-7 cells pretreated with SUMO-iRNAs were also infected with an adenovirus (Ad-Pdx1) to equip the cells with the ability to express Pdx1 (Fig. 3 and 4). Lysates from these cells were analyzed by Western blot analysis using both anti-Pdx1 and anti-SUMO-1 anti-
bodies. As shown in Fig. 3, A and B, SUMO-iRNAs reduced SUMO-1 expression by 60% of the control, and the expression of Pdx1 protein content was reduced by 80%. In contrast, no change was observed in β-actin levels in the presence or absence of SUMO-iRNAs (Fig. 3A). Additionally, Northern blot analysis showed that no change of the cellular content of Pdx1 mRNA was observed between the presence and absence of SUMO-iRNAs (Fig. 4, A and B). These findings show that the decreased expression of SUMO-1 causes a reduction of the Pdx1 protein contents. Because SUMO-1 does not affect Pdx1 mRNA levels, this observation suggests that SUMO-1 binds or conjugates with Pdx1.

Nuclear colocalization of SUMO-1 and Pdx1. To investigate whether Pdx1 could exist in the nucleus in the absence of endogenous SUMO-1 expression, we performed immunohistochemical analysis using both β-TC-6 and COS-7 cells. Both Pdx1 (Fig. 5A) and SUMO-1 (Fig. 5B) strongly colocalized in the nucleus of β-TC-6 cells (Fig. 5C). Because Pdx1 was expressed heavily in COS-7 cells without SUMO-iRNAs (Fig. 5D), we could not discriminate between the Pdx1 localization of nucleus and cytoplasm. However, SUMO-1 (Fig. 5E) exclusively expressed in the nucleus of COS-7 cells without SUMO-iRNAs. Thus the merged image for Pdx1 and SUMO-1 clearly revealed that both Pdx1 and SUMO-1 colocalized in the nucleus (Fig. 5F). However, low levels of SUMO-1 expression (Fig. 5H) were found in the nucleus of COS-7 cells in the presence of SUMO-iRNAs. In those conditions, Pdx1 (Fig. 5G) was mainly localized in the cytoplasm, with a little expression in the nucleus. The merged image revealed that both Pdx1 and SUMO-1 weakly colocalized in the cells (Fig. 5I). These data suggest that the sumoylation of Pdx1 was needed for Pdx1 to localize in the nucleus.

Effects of lactacystin (a proteasome inhibitor) on the stabilization of Pdx1. Sumoylation is believed to stabilize the adduct by inhibiting proteasomal degradation of ubiquitinated protein, and the converse, nonsumoylated Pdx1, is degraded in the proteasome. Therefore, we hypothesized whether nonsumoylated Pdx1 contents increased and were possibly detected by Western blot analysis when a proteasome inhibitor blocked the degradation of Pdx1 in the presence of SUMO-iRNAs to

Fig. 5. Immunocytochemical analysis showing nuclear and cytoplasmic localization of Pdx1 and SUMO-1 in β-TC-6 (A–C) and COS-7 cells transduced with Ad-Pdx1 in the absence (D–F) or presence (G–I) of SUMO-iRNAs. Cells were immunostained with anti-Pdx1/anti-Texas red antibodies (A, D, and G), and anti-SUMO-1/anti-FITC antibodies (B, E, and H). C, F, and I are overlapped images. Bar = 50 μm.
inhibit the formation of the adduct. To test this hypothesis, we exposed cells transfected with SUMO-iRNAs to 10 μM lactacystin, a proteasome inhibitor that blocks proteasome activity by targeting the catalytic β-subunit of the proteasome. In the cells transfected with SUMO-iRNAs alone, SUMO-1 protein expression decreased, and only the 46-kDa form of Pdx1 was detected, with a marked reduction of its content (Fig. 6L, lane 4, and 6L). However, the level of the 46-kDa form of Pdx1 was not significantly different between the presence and absence of SUMO-iRNAs by the addition of lactacystin (Fig. 6L). Additionally, the 46-kDa protein band increased in the presence of lactacystin compared with that in the absence of lactacystin (Fig. 6L, lanes 3 and 5, and 6L). As predicted, the cells, which were transfected with SUMO-iRNAs and exposed to lactacystin, showed the appearance of Pdx1 with a molecular mass of 34 kDa (Fig. 6L, lane 5). This is likely nonsumoylated Pdx1 protein. These results suggest that sumoylation of Pdx1 stabilizes this adduct by inhibiting its degradation by proteasomes.

Effects of insulin transcriptional activity on Pdx1 sumoylation. Finally, we tested the ability of sumoylated Pdx1 to stimulate insulin promoter activity by use of β-TC-6 and COS-7 cells. In this study, we used a luciferase assay comprised of the rat insulin promoter fused to the reporter to yield pINS-Luc. The activity of insulin gene expression was decreased significantly in β-TC-6 cells in the presence of SUMO-iRNAs (Fig. 7A). Consistently, COS-7 cells transfected with both SUMO-iRNAs and pcDNA-Pdx1 exhibited significantly lower transcriptional activity of insulin gene compared

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Fig. 6. A: Western blot analysis of Pdx1 and SUMO-1 in COS-7 cell extracts after the treatment of proteasome inhibitor (lactacystin). COS-7 cells were transfected with SUMO-iRNAs for 48 h, and then the cells were transduced with Ad-Pdx1 and incubated with 10 μM DMSO or lactacystin for 48 h. Whole cell extracts were prepared for 48 h of transduction with Ad-β-gal (lane 1) and Ad-Pdx1 (lanes 2 and 3). Lanes 4 and 5: results of Western blot analysis of COS-7 cell extracts with Ad-Pdx1 in the presence of SUMO-iRNAs. The abundance of β-actin reflects an equal loading of proteins in each lane. The blot shown is representative of 4 independent experiments. The positions of molecular mass are on right. B: data from densitometric analysis of the expression of the 46-kDa form of Pdx1. Values are shown as means ± SE (n = 4). *P < 0.05 vs. the values for COS-7 cell extracts transduced with Ad-Pdx1 in the presence of SUMO-iRNAs.

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SUMOYLATED Pdx1

A

![Graph A](image)

B

![Graph B](image)

with the cells transduced with pcDNA-Pdx1 alone (Fig. 7B). These results indicate that the modification by SUMO-1 is important for Pdx1 to exert its stimulatory activity on insulin gene transcription.

**DISCUSSION**

In this study, we found that Pdx1 was posttranslationally modified by SUMO-1. The addition of SUMO-1 to Pdx1 accounted for the 46-kDa form of Pdx1. Additionally, sumoylated Pdx1 enabled this form of the protein to localize in the nucleus and also prevented its degradation by proteasomes. Conversely, the non-sumoylated form of Pdx1 could not localize in the nucleus, and this inability to move into this compartment crippled its transcriptional activity on the insulin promoter. These results indicate that SUMO-1 modification is associated with the localization, stability, and transcriptional activity of Pdx1.

SUMO-1 is a 12-kDa protein of the ubiquitin family. The conjugation of SUMO-1 to proteins may regulate a number of transcription factors. Previous studies show that SUMO-1 regulates the subcellular localization of IκBα (5), stability of IκBα and p53 (4, 9, 29, 39), and transcriptional activity of the heat shock transcription factor 1 (14). However, there are no previous reports on the association of SUMO-1 with Pdx1. Thus we propose, based on our findings, SUMO-1 conjugation as a posttranslational modification of Pdx1. A potential mechanism by which SUMO-1 modification could regulate the Pdx1 localization was studied using immunoprecipitation (Fig. 2). Our results showed that Pdx1 was covalently attached to SUMO-1 in the nucleus. However, we could not conclude that sumoylation was directly needed for the nuclear localization and stability of Pdx1. In previous studies, a short consensus sequence, “KXE,” has been described as the SUMO-1 acceptor site in most of the known SUMO-1 substrates (14, 22, 29, 53). The “KKEE” amino acid sequence in the nuclear localization signal (NLS) of Pdx1 was mutated from lysine to arginine. However, sumoylation was observed in this mutant protein (data not shown), suggesting that Pdx1 was sumoylated by the different site of Pdx1 sequence from the classical consensus motif, KXE. Of course, there is another possibility, that SUMO-1 may regulate the function and stability of other proteins, which affect the nuclear localization and stability of Pdx1. Either SUMO-2 or SUMO-3 is speculated as candidate proteins that would affect the conjugation with Pdx1, since, like SUMO-1, SUMO-2/3 may play a similar role. SUMO-1 belongs to a family including SUMO-2 and SUMO-3 (19, 53), two proteins that are expressed in a wide range of tissues and cells (40). However, we found no evidence of either protein in association with Pdx1 in the present study (data not shown). All of these explanations are speculative; therefore, we need to further evaluate the exact motif of the sumoylation site of the Pdx1 molecule or the existence of other proteins that affect the nuclear localization and stability of Pdx1.

Our present study also showed that the 46-kDa form was the predominant form of Pdx1 present in both β-TC-6 and COS-7 cells transfected with pcDNA-Pdx1. This result is important because Pdx1 cDNA predicts it to have 283 amino acids with a molecular mass of 31 kDa (Fig. 1B). The possibility that phosphorylation accounted for the shift from the 31- to the 46-kDa form of Pdx1 was tested by exposing Pdx1 to phosphatase. The exposure of the phosphatase yielded a Pdx1 of slightly smaller molecular mass (Fig. 1B). These findings suggest that the shift of molecular mass of Pdx1 from 31 to 46 kDa was mainly explained by sumoylation of Pdx1.

Pdx1 is a homeodomain protein that binds to specific sites within the insulin gene promoter (34, 35, 44). This transcription factor also contains an NLS believed to be necessary for its transport in nucleus. The NLS is located within the homeodomain of Pdx1 and comprises seven amino acids (RRMKWKK; see Refs. 13 and 28). Nuclear translocation of Pdx1 is believed to be activated by signaling pathways through either PI 3-kinase or SAPK2/p38 (8, 20, 37, 51). Both pathways stimulate the phosphorylation of Pdx1 protein. However, the RRMKWKK motif does not contain serine, threonine, or tyrosine residues that may serve as po-
tentative phosphorylation sites. Therefore, there may be another site for phosphorylation in Pdx1, and the phosphorylation of NLS by itself is not necessary for the translocation of Pdx1 from the cytoplasm to the nucleus (28). Another phosphorylation site, except in NLS, can be related to the translocation of Pdx1. Nevertheless, previous reports (37, 42, 47, 48) are consistent with our present findings that Pdx1 is specifically localized in the nucleus of mouse insulinoma β-TC-6 cells without a shift of molecular mass by glucose concentration (2.5 and 25.0 mM; Fig. 1A), suggesting that the activation of nuclear Pdx1 translocation is also initiated by mechanisms other than phosphorylation.

Next, we wondered whether Pdx1 could localize in the nucleus without the association of SUMO-1. To solve this question, immunocytochemical detection of Pdx1 was performed using SUMO-iRNAs to suppress endogenous SUMO-1 expression. Recently, it has been shown that siRNA can be used in cultured mammalian cells (1, 7, 10). The delivery of short interfering RNAs (siRNAs) binds to a nuclease complex and forms an RNA-induced silencing complex that targets transcripts by base pairing between one of the siRNA strands and the endogenous mRNA. Here, we performed this method for efficient in vitro delivery of siRNAs to COS-7 cells and demonstrated effective and specific inhibition of SUMO-1 gene expression in the cells. In β-TC-6 cells, both Pdx1 and SUMO-1 expressed strongly in the nucleus and colocalized. Consistently, the merged image also revealed that both Pdx1 and SUMO-1 colocalized in the nucleus in COS-7 cells without SUMO-iRNAs. In contrast, in COS-7 cells transfected with SUMO-iRNAs, SUMO-1 was weakly found in the nucleus, and Pdx1 was mainly found in the cytoplasm (Fig. 5). It remains unclear whether Pdx1 conjugation with SUMO-1 is precedent for the nuclear translocation of Pdx1, but our data support the idea that SUMO-1 modification is needed to localize Pdx1 in the nucleus.

The biological consequences of the two processes are as follows: sumoylation and ubiquitination are quite different. SUMO-1 shares some identity with ubiquitin; both SUMO-1 and ubiquitin use lysine residues for the covalent conjugation to targets, and the steps of enzyme machinery leading to sumoylation and ubiquitination are mechanistically very similar (5, 15, 17, 36). Conjugation of SUMO-1 can increase stability, change subcellular localization, or affect interactions with partner proteins, but polyubiquitination of proteins is known to target selected proteins for proteolysis by the 26S proteasome (4, 9, 25, 29, 30, 38). Thus, another question prompted us to ask whether protein degradation by ubiquitination can occur rapidly in Pdx1 protein without SUMO-1 conjugation. To answer this question, we added a proteasome inhibitor, lactacystin, to inhibit proteolysis and SUMO-iRNAs to prevent endogenous SUMO-1 expression. Our results showed that only the 46-kDa form of Pdx1 was weakly detected in COS-7 cells transfected with SUMO-iRNAs alone. Additionally, the 46-kDa protein band from the cell extracts increased in the presence of lactacystin compared with that in the absence of lactacystin. We also found clear appearance of the 34-kDa form of Pdx1 in the presence of both lactacystin and SUMO-iRNAs (Fig. 6). These results suggest that nonsumoylated Pdx1 is degraded rapidly by ubiquitination in proteasome. Furthermore, it may be true that the Pdx1·SUMO-1 complex is also degraded in proteasome to some extent. The major results of this experiment indicate that the 46-kDa protein band is markedly reduced by the suppression of endogenous SUMO-1 expression and that double Pdx1 bands in molecular mass of 46 and 34 kDa are detected in the presence of both SUMO-iRNAs and lactacystin. Therefore, the 34-kDa band of Pdx1 is identified as a nonsumoylated one, indicating that Pdx1 sumoylation is prevented from degradation in the proteasome.

In summary, we have examined the transcription factor Pdx1 to understand its marked shift in molecular mass from 31 to 46 kDa. Although previous studies suggested that this change might be the result of phosphorylation of the protein, our findings show that the addition of SUMO-1 to Pdx1 is the more likely cause. The covalent attachment of SUMO-1 is associated with nuclear localization of Pdx1. Therefore, it prevents proteasomal degradation and also enables activation transcription of the insulin gene.

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